Non-Oxidative Enzymes of Spore Extracts

Hillel S. Levinson

Pioneering Research Division, Quartermaster Research and Engineering Center, Natick, Massachusetts.

MANY of the non-oxidative enzymes have already been mentioned, so I shall limit my discussion to those non-oxidative enzymes of spore extracts with which I have had recent personal experience. This limitation, you will discover, is very confining.

It is not unlikely that some of these enzymes may actually exist in an inactive condition within the spore, and that the process of grinding, etc., involved in preparation of extracts entirely distorts the true picture of the state of affairs within the spore. This warning should temper considerably your gullibility in accepting any theory which I may propound. Furthermore, you must realize that the evidence for some of these theories is far from overwhelming. Indeed, I am most grateful for this opportunity to speak more or less irresponsibly on a subject over which I have been mulling for some time. I have a great many slides but since each will appear only briefly, I trust you will bear with them.

In spite of the low metabolic activity of the spores, we felt rather early in our work that spores, as living things, must have some enzymes. One of the first enzymes with which we worked was the glutamic-aspartic transaminase of *Bacillus megaterium* spore extracts (Levinson and Sevag, 1954a). This enzyme (catalyzing the reaction: α-ketoglutarate + aspartate ⇋ glutamate + oxalacetate) was estimated by the measurement of oxalacetate production (Fig. 1). Briefly, the main finding was that, on a mg protein N basis, the glutamic-aspartic transaminase was as active in extracts of spores as in vegetative cell extracts of *B. megaterium*.

This finding—that is, the mere presence of enzyme in the spores—encouraged us to go on. However, before proceeding with the story, I must backtrack somewhat. Some of you may recall that we had shown a stimulation of germination and respiration of *B. megaterium* spores by manganous ion (Fig. 2). Incidentally, this figure gives an indication of the close relationship between germination, respiration, and turbidity of spore suspensions. Cobalt and zinc also had a stimulating effect on germination and respiration, but other cations, such as Mg^{++}, Ca^{++}, Cu^{++}, and Fe^{++} did not (Levinson and Sevag, 1953).

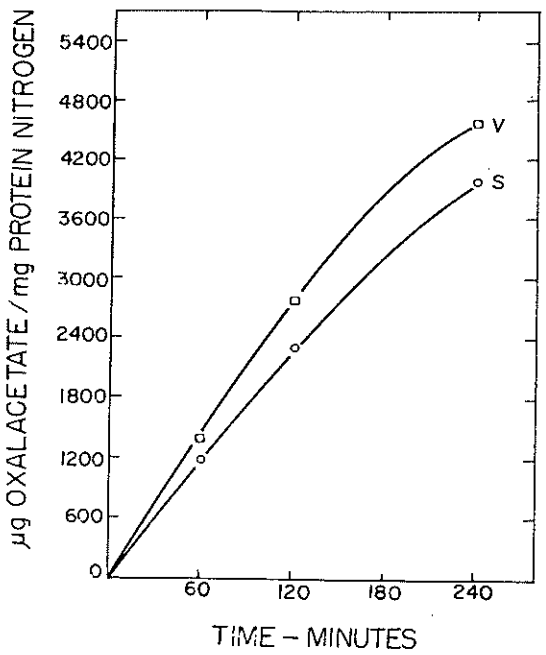

Fig. 1. Oxalacetate produced from α-ketoglutarate and aspartate via glutamic-aspartic transaminase of extracts of spores and vegetative cells of *B. megaterium* (Levinson and Sevag, 1954a).

We have had an idea that the effect of manganese is to activate an enzyme, which, within the spore, can produce substances useful to the spore in its germination. We have obtained certain indirect evidence supporting this point of view, which, by the way, is contradictory to the ideas expressed by Pulvertaft and Haynes (1951), who postulated the necessity for a specific excitant for spore germination. An extract of ground spores is stimulatory to germination of intact spores. The dialyzed extract is relatively inactive, but the material which passes through the dialysis bag is somewhat more active than was the original extract (Table I). In line with our theory, but not excluding other possibilities, we have postulated that this phenomenon may be due to continued production of the hypothesized essential principle during the course of the dialysis.

Spores will, as we all know, germinate in the absence of added manganese. However, it was determined by spectrographic techniques(Cohen and Wiener, 1954) that our spores, grown in the liver fraction medium suggested by Foster and Heiligman (1949), contained from 50 to 100 ppm of

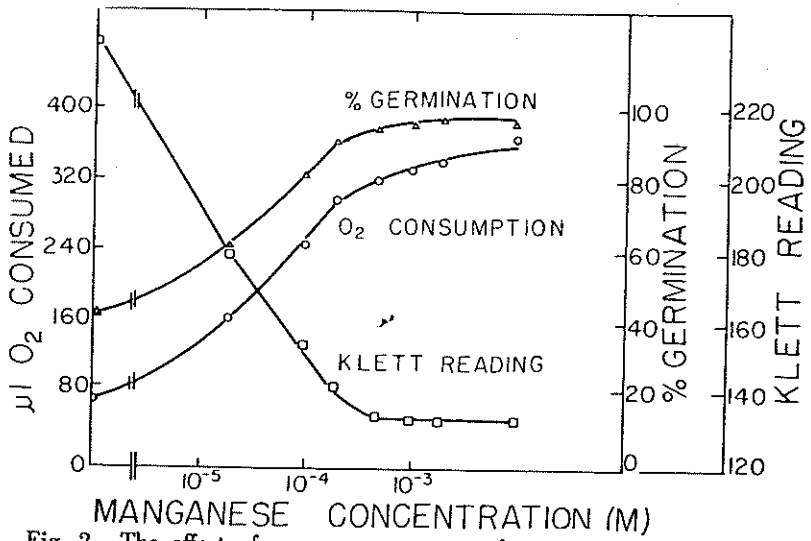

Fig. 2. The effect of manganese concentration on germination, oxygen uptake, and turbidity of spore suspensions. Indicated Klett readings represent turbidities. Spores were not heated. Manganese was as $MnSO_4$. Medium was composed of ammonium and potassium acetates, pH 6.6, 0.05M each; and glucose, 0.025M. Reaction period was 140 min. at 30°C (Levinson and Sevag, 1953).

manganese—an amount perhaps sufficient for activation of an enzyme necessary for the production of substances required for spore germination.

The list of enzymes activated by manganese (Table II) is incomplete and varied, and appears at first glance to be quite hopeless because of its very length and diversity. We thought it significant, however, that cobalt and zinc, which are peptidase activators, also stimulate *B. megaterium* spore germination. It may also be of significance that some of the proteolytic en-

TABLE I

Effect of spore extract on oxygen uptake of intact spores

Extract fraction	*O_2 uptake* (µl)
Whole extract	45
Dialyzable	105
Non-dialyzable	22
No extract	9

3.0 mg spores. Glucose 0.025M, 120 minutes incubation, 30°C. Potassium and ammonium acetates 0.15M, pH 6.8.

TABLE II

Some biological systems in which manganese is involved

System	Reference
1. Utilization of an enzyme precursor to benzoic acid oxidation.	Bernheim, 1954
2. Bacteriophage activity.	Rountree, 1955
3. Xylose isomerase of *Pasteurella pestis.*	Slein, 1955
4. Aldolase of *Brucella suis.*	Gary *et al*, 1955
5. Glutamine synthesis by *Micrococcus pyogenes.*	Fry, 1955
6. Activation of reproduction, sugar consumption, and respiration of *Saccharomyces cerevisiae.*	Lima, 1954
7. Enolase activation.	Malmström, 1953
8. Incorporation of formate into serine by pigeon liver extracts.	Sakami, 1955
9. Cysteine desulfurase of *Escherichia coli.*	Binkley, 1943
10. β-aspartokinase activation.	Black and Wright, 1955
11. Peptidase activation.	Smith, 1951

zymes are most active at elevated temperatures, and this may have something to do with the phenomenon of heat activation.

Hills (1949, 1950) has reported that L-alanine, a possible hydrolytic product of peptidases, is essential for the germination of *B. anthracis* and *B. subtilis* spores, and we have found, as have many others, that the germination of *B. megaterium* spores is much enhanced with L-alanine.

Another point in regard to the enhancement of germination by L-alanine is that spores immersed in L-alanine solution reach their maximal degree of germination practically instantaneously. When manganese is the stimulant, germination increases with the time of incubation (i.e., there is a definite lag). For example, a spore suspension which has germinated 60 per cent after 2 hours in manganese, or in L-alanine, will have reached this percentage within five to ten minutes in L-alanine, but it may take as long as 1.5 hours for this percentage of germination to be reached with manganese. This seems to indicate a more direct action of L-alanine than of manganese in germination.

Specifically, I believe that the spore creates its own stimulus for germination (providing a source of energy is available). No externally supplied excitant is needed. We have proposed that manganese acts, at least in part,

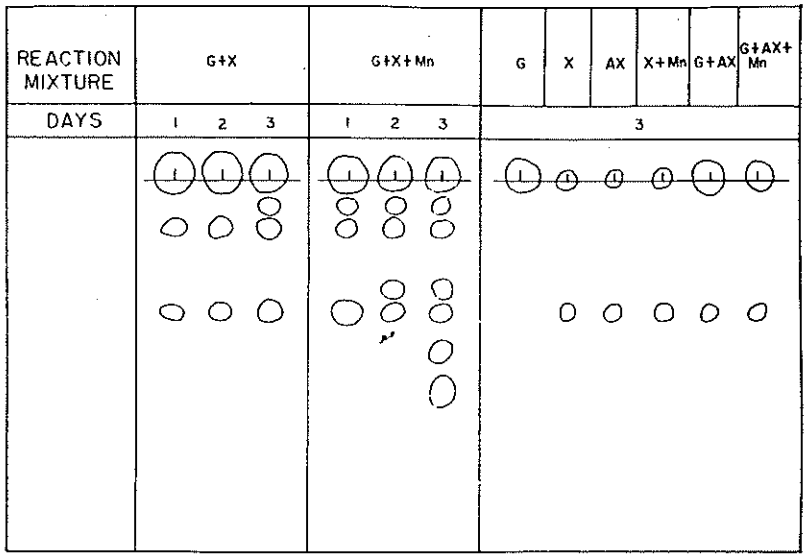

Fig. 3. Tracing of paper partition chromatogram showing hydrolysis of gelatin by spore extract. G = gelatin (1.0 per cent); X = spore extract (equivalent to 2.7 mg ground spores per ml reaction system); AX = autoclaved spore extract; Mn = manganese (10 ppm) as manganous sulfate. Mixtures chromatogrammed after 1, 2, and 3 days of incubation at 30°C (Levinson and Sevag, 1954b).

to activate protease or peptidase of the spores, which acting on spore material can produce amino acids or simple peptides which in turn stimulate the spore to germination. The site of action of these amino acids is another related problem which I am not prepared to discuss just now, but perhaps the L-alanine acts as Dr. Murty (this Symposium) has indicated.

We have some evidence that the spores of *B. megaterium* do contain proteolytic enzymes (Levinson and Sevag, 1954b). Using 1.0 per cent solutions of dialyzed gelatin and egg albumin as substrates, we have demonstrated the presence of proteolytic enzymes in spore extracts by several methods.

(1) *Paper partition chromatography for the detection of amino acids.* With gelatin as the substrate (Fig. 3), amino acids resulting from protein hydrolysis were observable after incubation of the protein with spore extract. The colors were much more intense when manganese was used, and in addition, the liberation of the amino acids from gelatin was accelerated with manganese. Much the same general picture is seen with egg albumin as the substrate (Fig. 4). Without manganese, there was no hydrolysis in

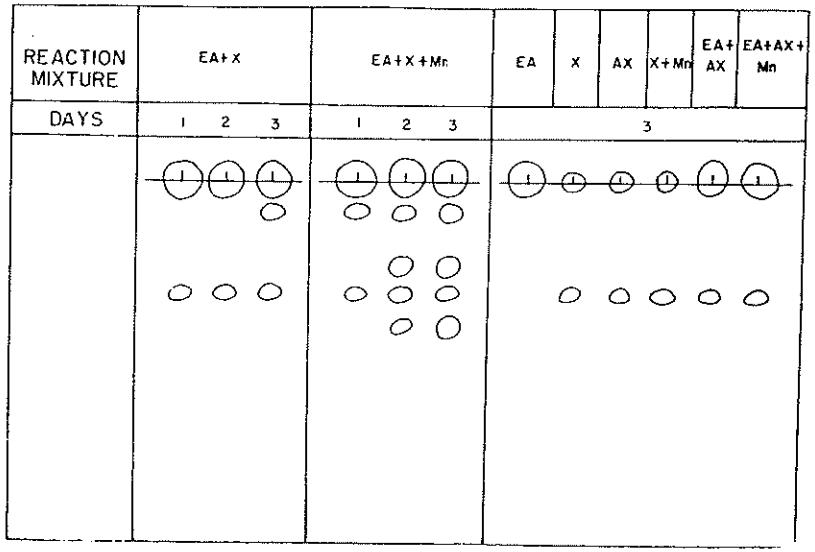

Fig. 4. Tracing of paper partition chromatogram showing hydrolysis of egg albumin by spore extract. EA = egg albumin (1.0 per cent); other conditions as for Fig. 3.

two days; but with manganese, definite signs of liberation of amino acids were observable in this time.

(2) *A colorimetric method based on the reaction of free amino groups with ninhydrin.* The proteinaceous substrate-enzyme mixture was incubated, treated with ninhydrin, and the resulting color was read in a Klett colorimeter. By this method, too, a breakdown of the protein substrate is evident, and manganese is decidedly stimulatory to this breakdown (Fig. 5). The question arises here as to whether manganese is an artifact. That is, is it possible that the addition of manganese to *any* peptide or amino acid mixture might increase the color developed with ninhydrin? Such does not appear to be the case (Table III).

TABLE III

Effect of manganese on ninhydrin reaction

"Amino Acid"	Manganese	Klett*
Sodium Glutamate	+	188
	—	185
Bacto Peptone	+	22
	—	25
DL-Alanine	+	104
	—	112

*The Klett reading indicates the intensity of the ninhydrin color.

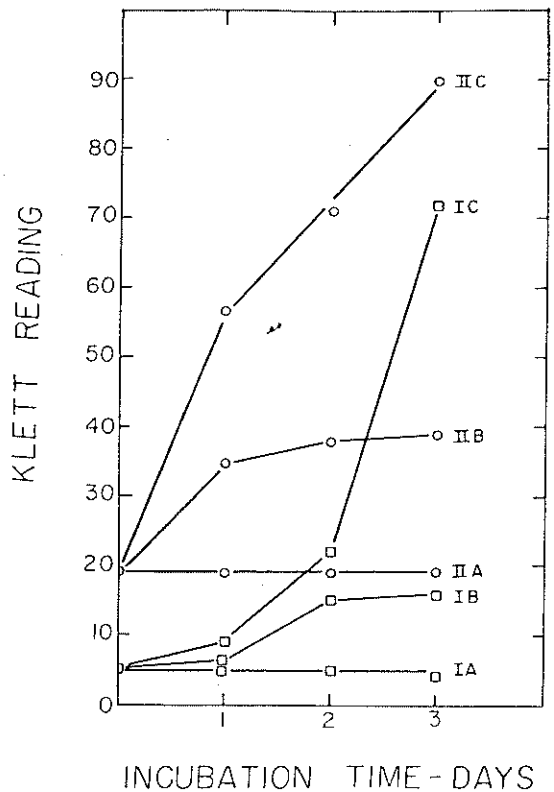

Fig. 5. Colorimetric estimation of proteolysis by spore extract. Curves labelled I represent data with gelatin as substrate. Curves labelled II represent data with egg albumin. A = autoclaved spore extract; B = spore extract; C = spore extract and manganese. Spore extract equivalent to 2.7 mg ground spores per ml reaction system. Incubation at 30°C (Levinson and Sevag, 1954b).

(3) *Viscosimetric method.* Changes in the viscosity of gelatin also give indication of proteolytic activity. All such experiments indicated that the rate of loss of viscosity with manganese was about 1.5 times that without Mn^{++} (Fig. 6).

Other pertinent information developed from the use of the uncentrifuged ground spores or homogenates. Incubation of the homogenates without the

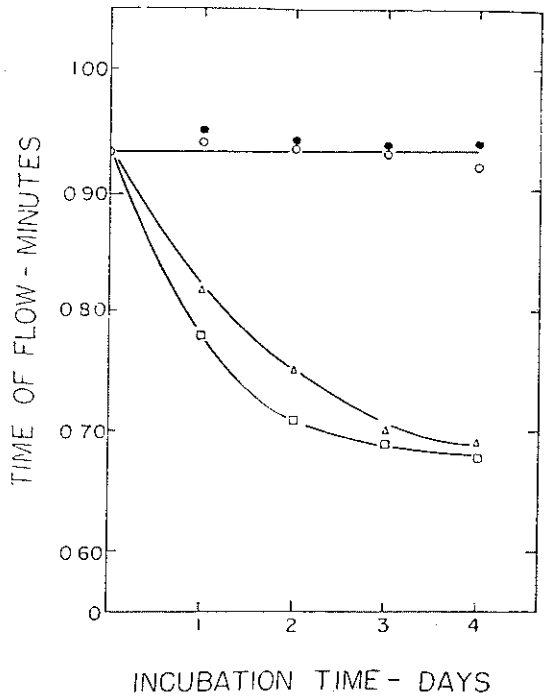

Fig. 6. Viscosimetric demonstration of gelatin hydrolysis by spore extracts. Open circles, gelatin and autoclaved extract; triangles, gelatin and spore extract; squares, gelatin and spore extract with manganese. Incubation at 30°C.

addition of other protein, resulted in an increase in the intensity of the ninhydrin color (Fig. 7). Not only that, but manganese accelerated and increased the proteolysis. This seems to us to be highly significant, since it could be evidence that the spore material itself can serve as substrate for the proteolytic enzymes of the spores. You may recall that in the proteolysis of gelatin and egg albumin by spore extract (Fig. 5) the incubation time was in days, and the Klett reading reached 90 in three days. In the case of these spore homogenates, however, the incubation period is in hours and the Klett readings go much higher. We attribute this faster proteolysis to the substrate's being, in this case, homologous for the enzymes concerned, thus permitting the breakdown to proceed much faster and to a greater extent than did the breakdown of the heterologous egg albumin and gelatin substrates.

As I have previously shown, spore extracts contain substances which

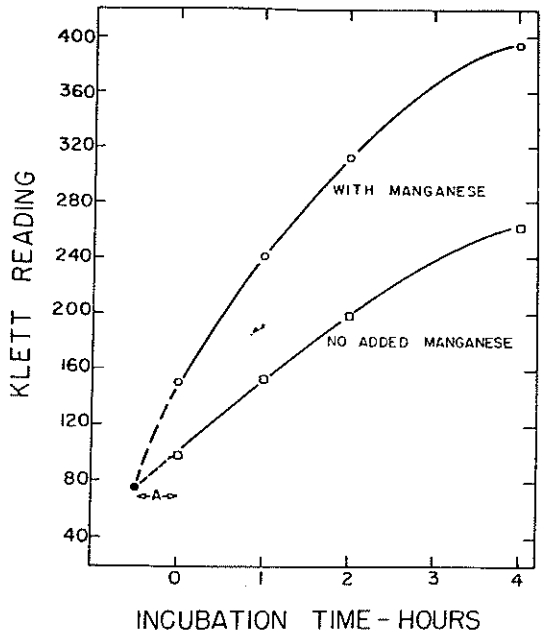

Fig. 7. Proteolysis in spore homogenates. Each ml of homogenate was derived from 30 mg ground spores. Ninhydrin color of homogenate developed after centrifugation following various times of incubation at 37°C. The closed circle indicates the estimated time of addition of manganous sulfate to one of the aliquots, and the distance, A, represents the time required for centrifuging. The dashed portions of the curves are, therefore, somewhat approximate. The magnitude of the Klett readings in comparison to those of Fig. 5 is due partly to the greater amount of spore material used, with consequently greater amounts of enzyme and substrate, but in greater measure, perhaps, to the homologous nature of the substrate (Levinson and Sevag, 1954b).

stimulate the germination of intact spores (Table I). These stimulating substances are dialyzable, as are amino acids. Chromatograms show the presence of several amino acids in the extracts—alanine and glutamic acid being the most noticeable. If we measure oxygen consumption as a criterion of germination, we find (Fig. 8) that we get similar curves of activity as a function of concentration when we use dialyzate of extract as when we use L-alanine. The stimulation obtained from 100 μg of extract solids was roughly equivalent to that obtained from 10 μg of L-alanine. Actually our extracts contain only about 5 μg L-alanine per 100 μg of extract solids. Thus, it is probable that the entire story of manganese stimulation does

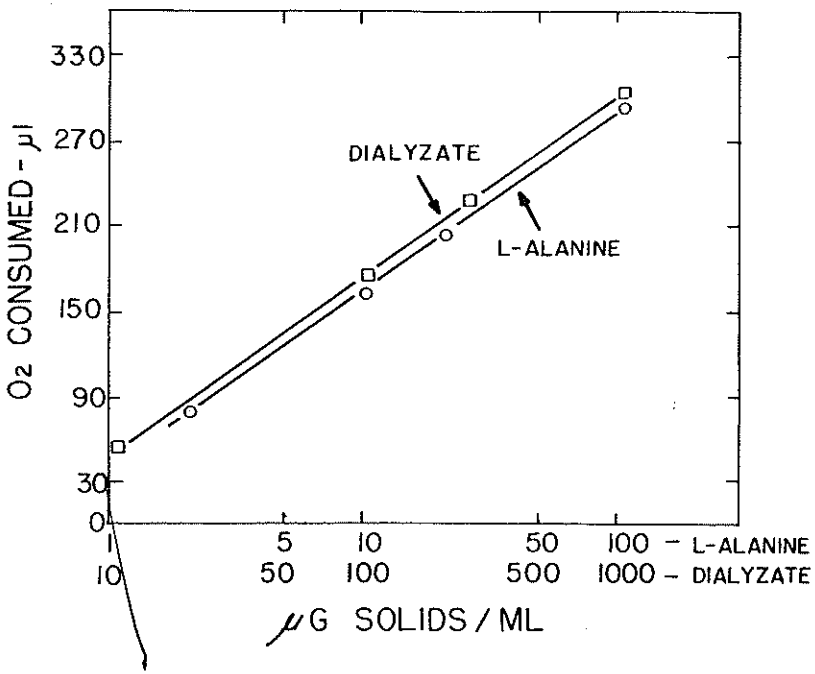

Fig. 8. The influence of L-alanine and of dialyzate of spore extract on oxygen uptake of intact spores. Oxygen uptake after 150 min. at 30°C in a medium containing ammonium and potassium acetates, 0.05M each, pH 6.8, and glucose, 0.025M.

not lie in the production of L-alanine. Indeed, we are sure of this on the basis of other experiments using D-alanine as an inhibitor of L-alanine activity (Levinson and Hyatt, 1955). We do feel, however, that these experiments gave us some insight into the problems involved in spore germination.

If the stimulation of germination of spores by spore extracts is due in appreciable part to the products of hydrolysis of spore protein, then one would expect more stimulation from extracts derived from incubated homogenates than from extracts made immediately after the preparation of the homogenate. Fig. 9 shows that this is the case.

Thus:

1. Spore extracts exert proteolytic activity.
2. Spore material can, under certain conditions, act as substrate for this activity.

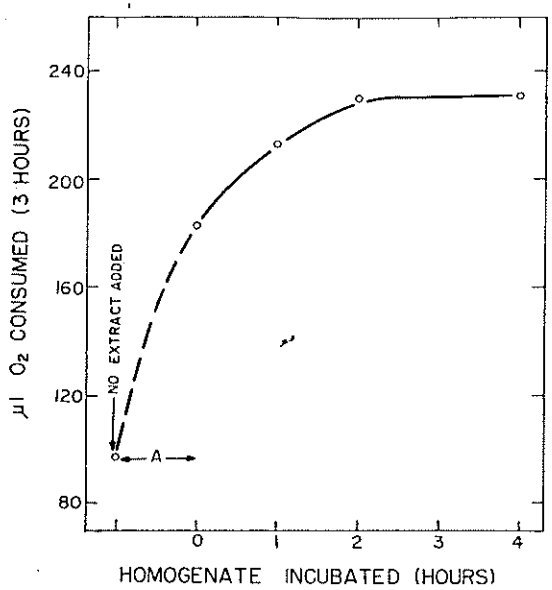

Fig. 9. Influence of time of incubation of homogenates on stimulation of oxygen consumption by extracts derived from them. Extracts prepared by centrifuging homogenates which had been incubated at 37°C for various periods of time. Reaction systems for oxygen consumption measurements contained ammonium and potassium acetates (pH 6.8, 0.05M each); glucose (0.025M); intact spores (3.0 mg); and extract equivalent to that derived from 2.5 mg spores.

3. Products of this hydrolysis of spore protein—L-alanine, for example —are capable of marked stimulation of spore germination.
4. Manganese stimulates both proteolysis and spore germination.

More recently, and none of this is published, we have been working with the enzyme pyrophosphatase in extracts of the spores of *B. megaterium*. As we test it, the enzyme catalyses the hydrolysis of sodium pyrophosphate to give orthophosphate:

$$Na_4P_2O_7 + H_2O \rightarrow 2\ Na_2HPO_4$$

There is not really much that I can say about the reaction. Its main interest to me lies in the observation (Fig. 10) that the activity of the enzyme is dependent on manganese. Without the addition of manganous ion we find either no activity or an extremely low activity, but with 5×10^{-4}M manganese we find nearly maximal activity. Cobalt and zinc also show a small amount of activation. The pH optimum (Fig. 11) for this manganese-activated pyro-

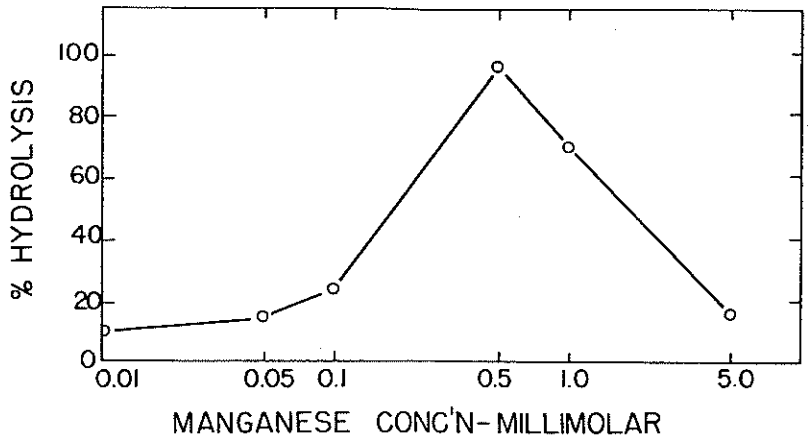

Fig. 10. The effect of manganous sulfate on pyrophosphatase activity of *B. megaterium* spore extract. Reaction systems contained 0.25 ml. of spore extract prepared in Mickle Disintegrator (25 mg spores per ml extract); 0.05 ml veronal buffer, 0.05M; 0.25 ml $Na_4P_2O_7$ (final concentration, 0.001M); and 1.0 ml of manganous sulfate. Reaction stopped by addition of 0.2 ml 100% tricholoroacetic acid after incubation for 30 min. at 37°C.

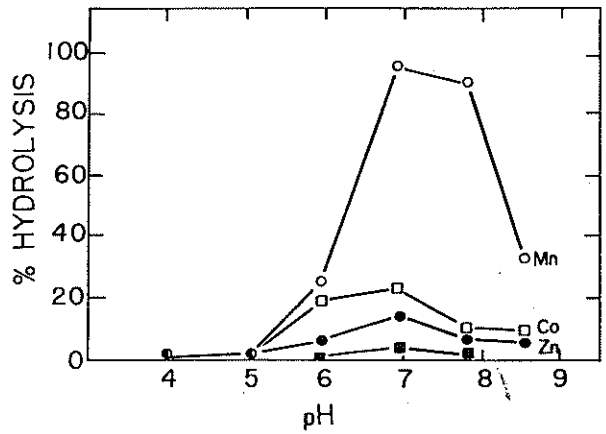

Fig. 11. pH-activity curve for spore extract pyrophosphatase. Conditions as for Fig. 10, except that acetate buffer (0.05M) was used at pH 4, 5, and 6. Divalent metal ions, as sulfates, in a final concentration of 5 x 10^{-4} M.

phosphatase is near neutrality, setting it off from the cobalt-activated acid pyrophosphatase and the magnesium-activated alkaline pyrophosphatase extracted from *Streptococcus faecalis* (Oginsky and Rumbaugh, 1955), both from the standpoint of the metal requirement and the pH optimum.

We have gotten all sorts of relationships in substrate concentration, enzyme concentration, etc., but these are not really significant now. I should like, though, to indulge in some wild conjectures as to the means by which this enzyme could be involved in spore germination.

This involves two important reactions. The first reaction is concerned with the synthesis of coenzyme A, and the second is concerned with transacetylation.

(1) ATP + Pantoic acid + β-Alanine ⇌ Pantothenic acid
+AMP +PP

(2) Ad. P~PP + CoASH + Acetate ⇌ Ad. P + CoAS~Ac + PP

Inorganic pyrophosphate is said by Dr. Fritz Lipmann (1954) to be a product in both reactions. In the presence of pyrophosphatase, the reactions will be forced to the right, due to the disappearance of the pyrophosphate, and the production of CoA and of acetyl CoA will be increased. These increases might result in more rapid utilization of glucose, with increased oxygen uptake. If these reactions can be shown to exist in spores, or in germinating spores, we may have a relationship between the requirements for manganese in spore germination and in pyrophosphatase activation. The possibility exists, and this is totally unsupported, that manganese acts as an activator of proteolytic enzymes resulting in the production of β-alanine, or of L-alanine convertible to β-alanine. This would fill a requirement for the production of coenzyme A. In addition, manganese would serve as a pyrophosphatase activator keeping the equilibrium of these reactions to the right. It is well known that the products of an enzymatic reaction can inhibit the specific enzyme involved in the reaction. Thus, α-amylase is strongly inhibited by α-maltose; fructose and glucose markedly inhibit invertase; cellulase is inhibited by cellobiose. Since inorganic phosphate is a product of the enzymatic hydrolysis of pyrophosphate, we might expect phosphate to inhibit pyrophosphatase. This indicates where we might get a glimmer of understanding of the oft-reported inhibition of germination by inorganic phosphate, i.e. through inhibition of pyrophosphatase. There is a suggestion of the actual involvement of pyrophosphatase in germination (Fig. 12), since after spores have germinated (i.e. become stainable), there is a great reduction in the amount of demonstrable pyrophosphatase. Extract of resting spores produces 1,350 μg of orthophosphate P per mg protein N in 10 minutes. Germinated spore extract produces 220 μg orthophosphate P in the same time, or about 16% as much as resting spore extract. It may

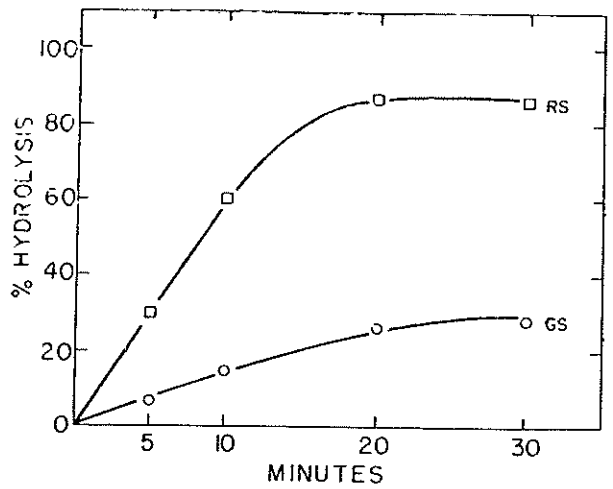

Fig. 12. Pyrophosphatase of extracts of resting spores (RS) and of germinated spores (GS). Spores ground in Mickle Disintegrator. RS ground after heating at 50°C for 15 min. GS prepared by incubation of RS with 0.025 M glucose for 1.5 hr. at 30°C (81% germination) followed by grinding. All mixtures contained 0.25 ml extract, 5 x 10^{-4}M manganous sulfate, 10^{-3} M $Na_4P_2O_7$, and veronal buffer at pH 7.0 in a total volume of 2.0 ml. Reaction stopped at indicated time by addition of 0.2 ml 100% trichloroacetic acid. 100% hydrolysis is calculated to yield 124.1 μg P.

be of significance that the extract of the so-called germinated spores was actually derived from spores which had germinated 81%, or to put it another way, 19% of whose spores had not germinated. Could this signify that only the resting spores had appreciable amounts of pyrophosphatase, and that the small amount of activity evidenced by the extracts of germinated spores was due to residual resting spores? This appears to be quite an unusual situation, and one which I believe is worth further investigation.

The spore coats of *B. megaterium* are rich in phosphorus which is acid and alkali insoluble. Fitz-James (1955) estimates that this P fraction is about 60% of the total P of *B. megaterium* spores. It is possible that this insoluble residue forms a lattice work making the spore coat impermeable to nutrients. The breakdown, or partial breakdown, of this lattice work through the mediation of the manganese-activated pyrophosphatase would permit nutrients to enter the spore and to participate in the biochemical events necessary for germination of the spore.

I realize that I have neglected some important non-oxidative enzymes. Perhaps Dr. Krask will touch on these. I hope that when the time for general discussion arrives we'll have comments on possible implications of the pyrophosphatase.

References

Bernheim, F. 1954. The effect of certain metal ions and chelating agents on the formation of an adaptive enzyme in *Pseudomonas aeruginosa*. Enzymologia *16:* 351-354.

Binkley, F. 1943. Serine dehydrase; cysteine desulfurase. J. Biol. Chem. *150:* 261-262.

Black, S. and N. G. Wright. 1955. β-aspartokinase and β-aspartylphosphate. J. Biol. Chem. *213:* 27-38.

Cohen, S. P. and D. A. Wiener. 1954. Spectrographic analysis of spores of *Bacillus megatherium*. Applied Spectroscopy *8:* 23.

Fitz-James, P. C. 1955. The phosphorus fractions of *Bacillus cereus* and *Bacillus megaterium*. II. A correlation of the chemical with the cytological changes occurring during spore germination. Can. J. Microbiol. *1:* 525-548.

Foster, J. W. and F. Heiligman. 1949. Mineral deficiencies in complex organic media as limiting factors in the sporulation of aerobic bacilli. J. Bact. *57:* 613-615.

Fry, B. A. 1955. Glutamine synthesis by *Micrococcus pyogenes* var. *aureus*. Biochem. J. *59:* 579-589.

Gary, N. D., L. L. Kupferberg, and L. H. Graf. 1955. Demonstration of an iron-activated aldolase in sonic extracts of *Brucella suis*. J. Bact. *69:* 478-479.

Hills, G. M. 1949. Chemical factors in the germination of spore-bearing aerobes. The effects of amino acids on the germination of *Bacillus anthracis* with some observations on the relation of optical form to biological activity. Biochem. J. *45:* 363-370.

Hills, G. M. 1950. Chemical factors in the germination of spore-bearing aerobes: observations on the influence of species, strain and conditions of growth. J. Gen. Microbiol. *4:* 38-47.

Levinson, H. S. and M. T. Hyatt. 1955. The stimulation of germination and respiration of *Bacillus megaterium* spores by manganese, L-alanine and heat. J. Bact. *70:* 368-374.

Levinson, H. S. and M. G. Sevag. 1953. Stimulation of germination and respiration of the spores of *Bacillus megatherium* by manganese and monovalent anions. J. Gen. Physiol. *36:* 617-629.

Levinson, H. S. and M. G. Sevag. 1954a. The glutamic-aspartic transaminase of extracts of vegetative cells and of spores of *Bacillus megatherium*. Arch. Biochem. and Biophysics *50:* 507-510.

Levinson, H. S. and M. G. Sevag. 1954b. Manganese and the proteolytic activity of spore extracts of *Bacillus megaterium* in relation to germination. J. Bact. *67:* 615-616.

Lima, U. de Almeida. 1954. Contribuiçao ao estudo da influencia do mangane sobre o desenvolvimento e atividades do *Saccharomyces cerevisiae*, Hansen. Ph.D. Thesis, University of Sao Paulo, Brazil.

Lipmann, F. 1954. On the mechanism of some ATP-linked reactions and certain aspects of protein synthesis. In Mechanism of Enzyme Action, ed. W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore. pp. 599-603.

Malmström, B. G. 1953. Interaction of manganous ions with enolase. Nature *171:* 392-393.

Oginsky, E. L. and H. L. Rumbaugh. 1955. A cobalt-activated bacterial pyrophosphatase. J. Bact. *70:* 92-98.

Pulvertaft, R. J. V. and J. A. Haynes. 1951. Adenosine and spore germination; phase contrast studies. J. Gen. Microbiol. *5*: 657-663.

Rountree, P. M. 1955. The role of divalent cations in the multiplication of staphylococcal bacteriophages. J. Gen. Microbiol. *12:* 275-287.

Sakami, W. 1955. The biochemical relationship between glycine and serine. In Amino Acid Metabolism, ed. W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore. pp. 658-683.

Slein, M. W. 1955. Xylose isomerase from *Pasteurella pestis*, strain A-1122. J. Am. Chem. Soc. *77:* 1663-1667.

Smith, E. L. 1951. The specificity of certain peptidases. Advances in Enzymology *12:* 191-257.

Reprinted from Spores, edited by H. Orin Halvorson, published by American Institute of Biological Sciences, 2000 P St., N.W., Washington 6, D. C.